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Full Length Article

Antibacterial Mode of Action of Grapefruit Seed Extract against Local Isolates of Beta-Lactamases-Resistant *Klebsiella pneumoniae* and its Potential Application

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Abstract

Grapefruit seed extract (GSE) has attracted wide attention through its use as a supplement in food manufacturing. This study shows that GSE is a potential antibacterial agent that distorts the cell membrane and causes a wide range of inhibitory effects in *Klebsiella pneumoniae*. Remarkably, GSE was able to inhibit growth and decrease biofilm formation of clinical isolates of *K. pneumoniae*. TEM images showed significant damage to cells treated with GSE, which results in the leakage of the cytoplasmic component. Plasmid resistance gene bla_{TEM} was detected in two isolates, whereas three isolates were found to harbor the bla_{CTX} plasmid resistance gene. Both genes were degraded enormously by the action of 10% of GSE. Lastly, GSE exhibited bacterial clearance and anti-adhesion activity using Band-Aids, whereas, no notable reduction was seen in the case of catheters. The results here offer a foundation for developing an effective therapy using GSE against clinical isolates of *K. pneumoniae*.

Keywords: Grapefruit seed extract; *Klebsiella pneumoniae*; Antibiotic resistance; Virulence plasmids

Introduction

Advances in antibiotic development have greatly increased the probability of survival greatly, enabling procedures such as chemotherapy, surgical operations, and childbirth to proceed effectively (Sedighi *et al*. 2017). On the other hand, decreased efficiency of available antibiotics has led to the rise of antibiotic-resistance, which has quickly become an alarming concern in the developing world. Simultaneously, the spread of multidrug-resistant (MDR) bacteria are significantly limiting treatment options for infections (Lin *et al*. 2016), causing an amalgam of complications such as prolonged illness and hospitalization, increased healthcare bills, risks for contracting other related infections, and death. Bacteria resist antibiotics in several ways; these may include the formation of physical barriers such as biofilms, modification of antibiotic target sites, expression of influx/efflux pumps, genetic mutations and adaptations, altered outer membrane permeability, and acquisition of resistance genes or drug-inactivating enzymes (Paterson and Bonomo 2005; Tenover 2006).

Finding new antimicrobial drugs against drug-resistant Gram-negative bacterial infections are extremely problematic, owing to the complex structure of their cell wall (Freitas *et al*. 2013). Antibiotic resistance in Gram-negative MDR bacteria are most often plasmid-mediated, marking their territory on the World Health Organization's (WHO) critical priority pathogens list (WHO 2019). Opportunistic β-lactamase-producing *K. pneumoniae* strains have been identified as the cause of numerous community and hospital-acquired infections, such as pneumonia, sepsis, urinary tract infections, meningitis, and soft tissue infections (Paterson and Bonomo 2005; Lin *et al*. 2016). They are particularly seen in the elderly, immunocompromised patients, and those with indwelling medical devices in healthcare facilities. These strains contain plasmids that harbor resistance and virulence genes, enabling resistance against *β*-lactam antibiotic drug-of-choices used for treatment, such as penicillins, cephalosporins, carbapenems, aminoglycosides, or fluoroquinolones *via* the production of extended-spectrum Beta-lactamases (ESBLs); enzymes that inactivate β-lactam antibiotics by hydrolysis (Paterson and Bonomo 2005; Tenover 2006). Prevalent *β*-lactamases in *K. pneumoniae* strains such as in bla_{CTX}, are resistant against cefotaxime, while in bla_{TEM}, they are more effective against ampicillin and oxyimino-*β*-lactams groups such as ceftazidime, cefotaxime, and aztreonam (Ramirez *et al*. 2014; Sedighi *et al*. 2017). Other virulence factors contributing to pathogenicity include capsular polysaccharide, lipopolysaccharide, siderophores, and fimbriae, which allows the cells to grow on the surfaces of medical devices, and infected tissues as biofilms (Ramirez

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et al. 2014).

A wide variety of metabolites localized in plant products are known to exert strong antimicrobial properties, including tannins, alkaloids, and flavonoids (Gupta and Birdi 2017). There is potential within GSE, a commercially available natural product derived from the seed and pulp of grapefruits. A previous report has stated that the majority of polyphenolic compounds in GSE are limonoids and flavonoids (Jang *et al*. 2011). The main bioactive flavanone compound in GSE is naringin, which is present in large amounts. On the other hand, the flavonols hesperidin and quercetin (Table 1) exist in low concentrations (Panche *et al*. 2016; Han *et al*. 2021). GSE has been conclusively reported to possess strong antimicrobial and antifungal properties, whilst also disrupting the bacterial membrane of some gram-positive and gram-negative organisms (Reagor *et al*. 2002; Cvetnić and Vladimir 2004; Choi *et al*. 2014). Another study has evaluated the inhibitory effect of GSE against biofilms (Song *et al*. 2019). Further applications of GSE were used for the preservation of minimally processed vegetables (Xu *et al*. 2007) and used as a composite film in the area of food technology (Tan *et al*. 2015). However, to our knowledge, no reports have demonstrated the mode of action of GSE against plasmids harboring resistance genes in *K. pneumoniae*. Therefore, this study further sheds light on the GSE mechanism of action against *K. pneumoniae* by investigating virulence-associated properties, including cell adherence, biofilm production, and the presence of antibiotic-resistant genes.

Materials and Methods

Preparation of sample

Nutribiotic® GSE liquid concentrate was purchased; according to the manufactured product, this extract is comprised of vegetable glycerin (67%) and grapefruit seed extract (33%). GSE was diluted to several concentrations with sterile distilled water for the following *in vitro* experiments, and the solutions were referred to as 5, 7.5, 10, 50, 75 and 100% v/v GSE, respectively.

Bacterial isolates

Clinical isolates of *K. pneumoniae* (n = 11) used in the present study were obtained from various local hospitals in Sulaimani, Kurdistan Region of Iraq. All *K. pneumoniae* isolates were isolated from urine specimens and collected during September and October of 2018. Each isolate was grown in nutrient broth at 37°C for 24 h. Purity of the isolates were confirmed by plating on MacConkey agar and identified using VITEK 2 (bioMérieux, USA).

Antimicrobial susceptibility testing

Antimicrobial susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines using the Kirby- Bauer disk diffusion method (Humphries *et al*. 2018). The following antibiotics were used: Cefotaxime (CTX: 30 μg), Ciprofloxacin (CIP: 10 μg), Ampicillin (AMP: 25 μg) and Ceftriaxone (CRO: 10 μg). The turbidity of each bacterial suspension was adjusted to match (10^8 CFU/mL), and 100 μL of culture was spread on Müeller-Hinton agar plates while the antibiotic discs were impregnated on the bacterial inoculum. Plates were then incubated at 37°C for 24 h, and the zones of inhibition were measured. The sensitivity was compared according to literature (Balouiri *et al*. 2016).

Agar diffusion assay

The antimicrobial activity of GSE was conducted on Müeller-Hinton agar plates using the agar well diffusion assay with a few modifications (Ahmed *et al*. 2018). Eleven isolates of *K. pneumoniae* were grown in nutrient broth at 37°C for 24 h. The number of cells was adjusted to match 10^8 CFU/mL in a standard plate count procedure. One hundred microliters of inoculum were dispersed over the entire surface of Müeller-Hinton agar plates. Four wells were made in each plate using a sterile glass Pasteur pipette while 100 µL of distilled water as control, along with 50, 75 and 100% GSE were loaded into the specific wells. Plates were incubated at 37°C overnight and inhibition zone diameters (mm) were measured. Antimicrobial activity was expressed based on the average mean and standard deviation of triplicate results.

Minimum inhibitory concentration (MIC) assay

To determine the lowest concentration of GSE that inhibits bacterial growth, MIC was performed using the microtiter broth dilution method (Balouiri *et al*. 2016) with a few modifications. Eighty microliters of GSE dilutions (0, 5, 7.5, 10, 25, 50 and 100%) were distributed in 96-well microtiter plates as well as a positive control (containing broth and bacteria only) and negative control (containing broth and sample only). Finally, each well was inoculated with 120 µL of bacterial suspension (10^8 CFU/mL) and the microdilution trays were incubated at 37°C overnight under a gentle shaking in the microplate incubator-shaker PST-60 HL Plus (BOECO, Germany). The optical density (OD) at 600 nm was measured using a microplate spectrophotometer (Biotech µQuant, USA). A total of 3 experiments were performed.

Antibiofilm assay

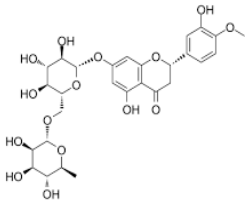
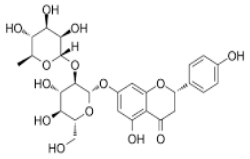
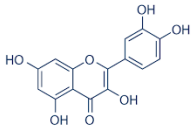
Biofilm formation of 11 isolates of *K. pneumoniae* was studied by tissue culture plate (TCP) method previously adopted by (Hamzah *et al*. 2018). Fresh overnight bacterial culture in nutrient broth was adjusted to be 10^8 CFU/mL. Cultures of 200 μL were placed in 96-well microtiter plates

followed by incubation for 24 h at 37°C under gentle shaking in the microplate incubator-shaker PST-60 HL Plus (BOECO, Germany). After incubation, the contents in the wells were discarded and rinsed 3 times with 200 µL phosphate buffered saline (PBS, pH 7.2) to remove free floating bacteria. After drying, the adherence of sessile bacteria was fixed with sodium acetate (2%) and stained with crystal violet (0.1%, w/v) for 30 min. After staining, the liquid was discarded and rinsed 3 times with distilled water. The plate was then allowed to dry at room temperature for about an hour, after which 200 μL of (95%) ethanol was added to the wells to solubilize the stains. The absorbance at 595 nm was measured via microplate spectrophotometer (Biotech µQuant, USA). This experiment was performed 3 times to compare and analyze the average of each result. The same method was employed to observe biofilm reduction by GSE using different dilutions. Simultaneously, 5 µL was taken from each well and streaked on nutrient agar and Congo Red Agar (CRA) plates, followed by incubation at 37°C overnight. Briefly, CRA was prepared as followed: brain heart infusion (BHI, 37 g/L), sucrose (50 g/L) and agar (10 g/L) were prepared and autoclaved at 121°C for 15 min. Congo red dye (Sigma-Aldrich, Germany) (0.8 g/L) was also prepared simultaneously and added to warm (50°C) BHI agar. Color and texture of the colonies were then analyzed to evaluate biofilm efficacy (Kalishwaralal *et al*. 2010)

Optical and transmission electron microscope (TEM)

Bacterial cells were observed with light microscope both before and after treatment with GSE. Sterile coverslips were cut into small pieces and introduced into two wells on a 25-well microtiter plate; one well containing only a bacterial culture of *K. pneumoniae* as control, and the other containing *K. pneumoniae* culture treated with 10% GSE. The microtiter plate was then incubated overnight at 37°C. Next, the coverslips were taken out of the wells and Gram stained to observe both treated and untreated cells, while the cells on the other side of the coverslip were wiped by a paper towel. For TEM, bacterial cell suspension with an absorbance of 0.2 was prepared in nutrient broth, treated with 10% GSE (v/v), and incubated at 37°C for 20 h. Samples were then sent to the Al-Nahrain University, Baghdad, Iraq to be observed on a transmission electron microscope (Phillips CM10, Holland), according to the modified protocol by (Cornelissen *et al*. 2018). Manual staining was performed as a drop of bacterial suspension was added to a TEM grid mesh, followed by the addition of a drop of uranyl acetate solution for 10 min, ideal for ultra-fine sections and negative staining. The grid was then rinsed with distilled water to remove any residual and unbound stains. After drying, the grid was coated with a formvar support film, and viewed on the TEM. The image was captured via (Hamamatsu Orca, Japan).

Table 1: Bioactive flavonoids compounds in GSE

Flavonoid	Chemical formula	Flavonoid subclass
Hesperidin		Flavanone
Naringin		Flavanone
Quercetin		Flavonols*

*Flavonols are flavonoids with a ketone group

Protein leakage assay

The effect of GSE on membrane damage was studied by quantifying the leaked cytoplasmic proteins. Protein leakage from bacterial cells was determined using the A280 assay (Miksusanti *et al*. 2008). Briefly, bacterial cell suspension with an absorbance of 0.2 was prepared in nutrient broth and treated with 10% GSE (v/v) and incubated at 37°C for 20 h. Samples were then centrifuged at 4,000 rpm for 5 min using the centrifuge 5702 R (HERMLE Z200A, Germany), and supernatants were subjected for protein quantification using the NanoDrop 2000 (ThermoFisher, United States). Untreated bacterial cells were also used as controls.

PCR amplification

The polymerase chain reaction (PCR) method was performed to detect two antibiotic resistant genes of bla_{CTX} and bla_{TEM} localized in specific plasmids of *K. pneumoniae.* Novel primers were designed based on data from plasmids-mediated resistance genes collected from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore). A total of 40 antibiotic resistant gene sequences of different *K. pneumoniae* strains were collected from the NCBI nucleotide database. Specific primers were designed based on the multiple sequence alignment of bla_{CTX} and bla_{TEM} genes of 40 strains of *K. pneumoniae* submitted to NCBI using Clustal Omega. The sequences of the selected primers are shown in Table 2. PCR reaction mixture set-up contained 10 μL master mix, 1 μL of forward and reverse primers each, 1 μL of DNA, and 7 μL of distilled water totalling in 20 μL. The PCR protocol for 30 cycles was as follows: an initial denaturation at 94°C for 2 min, then 94°C for 30 s, 50°C for 30 s, 68°C for another 30 s, followed by a

Table 2: Primer sequences for detection of *bla*$_{CTX}$ and *bla*$_{TEM}$ in *K. pneumoniae* isolates

Primer name	Primer sequence (5'-3')	Amplicon size (~ bp)	Antibiotic resistant
blaTEM_F	GATCCTTGAGAGTTTTCGCC	530	Ampicillin
blaTEM_R	GCAGAAGTGGTCCTGCAACT		
blaCTX_F	AGACTGGGTGTGGCATTGAT	600	Cefotaxime
blaCTX_R	CCAGGAAGCAGGCAGTCC		

*In the following primers, F denotes forward and R denotes reverse

Table 3: Antimicrobial susceptibility of *K. pneumoniae* isolates to different antibiotics

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive (%)
Ciprofloxacin	36%	-	64%
Ceftriaxone	64%	18%	18%
Cefotaxime	64%	9%	27%
Ampicillin	82%	-	18%

*Antibiotics were chosen according to the resistant genes on *bla*$_{CTX}$, *bla*$_{TEM}$

final extension at 68°C for 7 min. The PCR product was analysed by electrophoresis in a 1% agarose gel in TAE buffer at 90V for 20 min, stained with ethidium bromide, and the image was captured *via* MultiDoc-It™ Imaging System (UVP, USA).

Anti-adhesion assay using Band-Aids and catheters

GSE was evaluated for its efficiency using applications with the surfaces of Band-Aids and catheters. Sterile Foley catheters and Band-Aids were purchased from the market and cut into uniformly sized sections of 6–7 mm. The pieces were soaked in 100% GSE and kept overnight at 40°C to facilitate impregnation of GSE on the Band-Aid and catheter surfaces (Halawani 2017). Next, the pieces were soaked in 200 μL of *K. pneumoniae* cultures in a 96-well microtiter plate and incubated at 37°C overnight. Untreated Band-Aid and catheter pieces immersed in bacterial cultures served as controls. The pieces of Band-Aids and catheters from the microtiter plate were taken out and washed with 1X PBS (phosphate buffered saline, pH 7.2) to remove free floating bacteria from the surfaces. The pieces were then allowed to sit in PBS for 30 min. Serial dilutions were made from this suspension and 100 μL was then evenly spread on the entire surface of a nutrient agar plate and incubated at 37°C overnight. Plates were then counted to observe the number of colonies before and after treatment with GSE (Tan *et al*. 2015; Hixon *et al*. 2017).

Statistical analysis

Microsoft Excel (Microsoft 2016) was used for all statistical computations. Three independent measurements of each experiment mentioned above were pooled and subjected to statistical analysis.

Results

Antimicrobial susceptibility profile

In total, 11 isolates of *K. pneumoniae* were retrieved and their antimicrobial resistance profile against 4 different antimicrobial agents was tested (Table 3). Among them, 64% were found to be resistant to ceftriaxone, while 18% were intermediate and sensitive to it. 82% were resistant to ampicillin, however only 18% were sensitive to it. Similarly, 64% were resistant to cefotaxime and 36% to ciprofloxacin, while 27% were sensitive to cefotaxime and 64% to ciprofloxacin.

Determination of antimicrobial activity and MIC of GSE

To investigate the argument that the presence of preservatives was responsible for the antimicrobial properties of GSE, 67% of glycerin was evaluated in a well-diffusion assay. Glycerin was tested against Gram-negative and Gram-positive isolates. Out of all the bacterial isolates tested, glycerin shows no zones of inhibition (data not shown). Similarly, GSE was screened for its antibacterial efficacy. The antibacterial activity of three GSE concentrations (50, 75 and 100%) was determined by measuring the zone of inhibitions against 11 *K. pneumoniae* isolates, as represented in Table 4. All 3 concentrations exhibited antibacterial activity against the tested bacteria, with the diameter of inhibition zones ranging from 8.3 to 17.3 mm, respectively.

The MICs of grapefruit seed extract against the tested bacteria was determined by broth microdilution method. The MIC values ranged between 7.5 and 10% (v/v). At 10%, no growth was observed. Spot assay on NA plates was also performed, whereas bacterial growth is seen at 0% GSE (control) and inhibited at 10% GSE.

Biofilm inhibition and degradation effects of GSE

The inhibitory effect of GSE on biofilms formed by *K. pneumoniae* isolates was performed by the Tissue Culture Plate method (Fig. 1). Nine out of eleven (81%) isolates were found to be biofilm producers, where 3 were strong biofilm producers, 5 were moderate, and 1 was weak. GSE inhibited the formation of biofilms at 5%.

Table 4: Antimicrobial activity of GSE against *K. pneumoniae* isolates

Bacterial code	Zone of inhibition (mm)*		
	GSE concentration %		
	50%	75%	100%
Kp1	8.3 ± 0.6	10.3 ± 0.6	11.3 ± 0.6
Kp2	10.0 ± 1	12.3 ± 2.3	17.3 ± 1.2
Kp3	8.7 ± 0.6	10.7 ± 0.6	11.7 ± 1.5
Kp4	8.7 ± 0.6	11.0 ± 1.0	12.7 ± 1.5
Kp5	8.3 ± 0.6	10.7 ± 3.8	11.3 ± 1.5
Kp6	8.7 ± 1.2	10.7 ± 1.5	12.0 ± 1
Kp7	8.7 ± 0.6	10.7 ± 0.6	13.0 ± 0.0
Kp8	9.0 ± 1	9.3 ± 2.1	10.3 ± 1.5
Kp9	8.7 ± 0.6	10.0 ± 1.0	11.3 ± 1.2
Kp10	7.7 ± 0.6	9.3 ± 0.6	11.0 ± 1.7
Kp11	9.1 ± 1	11.3 ± 2.3	12.7 ± 2.1

*Determined by the diameter of inhibition zones (mm) using the agar well diffusion method

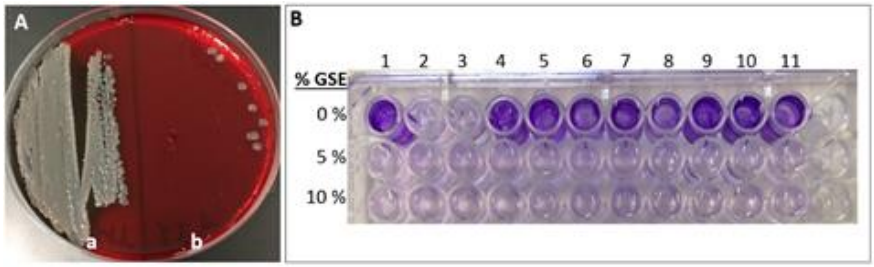


Fig. 1: Biofilm reduction in *K. pneumoniae* by GSE. **A.** CRA plate (a: colonies of strong biofilm-producing isolates as indicated by crystalline colonies-turned black. b: reduced biofilm formation by treatment with 5% GSE). **B.** The cells that adhered to the plate after washing were visualized by fixing with sodium acetate (2%) and staining with crystal violet (0.1%, w/v). Absorbance was measured at 595 nm

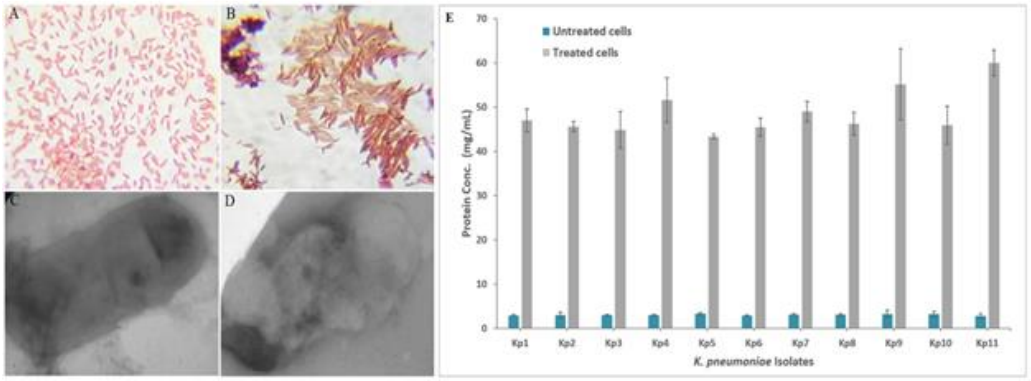


Fig. 2: Effect of GSE on *K. pneumoniae* cells. **A** and **B** represent Gram stain (**A** represents cells before treatment and **B** after treatment with 10 % GSE). **C** and **D** represent TEM micrographs (**C** represents the cell before and **D** after treatment with GSE). **E**. Protein leakage of *K. pneumoniae* isolates by GSE (The bars represent mean of three data points and SD (Mean ± SD)

Analysis of *K. pneumoniae* cells using TEM and protein leakage

Light and TEM microscope revealed the cell morphology of *K. pneumoniae* cells before and after treatment with 10% GSE. As seen in Fig. 2 (A and B), appearance of cells prior to treatment were apparently normal bacilli; whereas in the presence of 10% GSE, treated cells appeared to be distorted and changed in length, shape, and size. Similarly, TEM shows (Fig. 2C–D) that after treatment with GSE, evident signs of cell damage and disruption of membrane such as ruptured lines on cell surfaces, cell debris, intracellular damage, cell leakages, and shrinkage can be seen.

Leakage of proteins from the bacterial cells of 11 *K. pneumoniae* isolates was analyzed after 20 h by measuring the absorbance at 280 nm (Fig. 2E). Substantial differences are observed in leaked protein amounts before and after treatment with 10% of GSE. The amount of leaked proteins from untreated cells ranges within 2.95–3.29 mg/mL, while ranging within 43.29–59.99 mg/mL from cells treated with 10% GSE. Maximum protein leakage can be seen in isolate Kp11, at 59.99 mg/mL.

Analysis of antibiotic-resistant genes by PCR amplification

Plasmid analysis of the clinical *K. pneumoniae* isolates showed the presence of 2 plasmids with size range from 0.8 and 1.3 kb (data not shown in this study). As seen in Fig. 3, PCR was carried out to amplify resistant genes bla_{CTX} and bla_{TEM}. Among the 11 examined isolates, three (27%) were positive for blaCTX (product size ~ 600 bp) and two (18%) isolates for blaTEM (~ 530 bp) genes, respectively. Both plasmid genes were degraded by treatment with GSE.

Applications of GSE with Band-Aids and catheters

The anti-adhesion activity of GSE was assessed against selected adherent *K. pneumoniae* bacterial isolates on the surfaces of Band-Aids and catheters (Fig. 4). Adhered bacterial cells (CFU/ mL) were compared before and after treatment with GSE, with significant reduced adhesion seen in the case of treated cells on the surface of Band-Aids. No notable reduction was seen in the case of catheters.

Discussion

Plant products are widely known to contain a diverse variety of phytochemicals and secondary metabolites that exert strong antimicrobial properties. Naringin seems to be the main substance that exhibits a favorable antibacterial effect in GSE (Han *et al*. 2021). According to previous reports, GSE exhibits strong antimicrobial activity against bacteria (Reagor *et al*. 2002), and fungi (Choi *et al*. 2014). To our knowledge, this is the first study investigating the use of GSE in degrading plasmid-mediated ESBLs genes in *K. pneumoniae*. Eventually, the results of this study support the hypothesis that GSE can drive multiple inhibition mechanisms against *K. pneumoniae*, with equal or greater efficacy as compared to other choices of *K. pneumoniae* antibiotics-like substances. Successful antigrowth and antibiofilm activities of GSE are observed against gram-positive and gram-negative isolates (data not shown in this study). Additionally, the study has been designed to assess the mode of action against the bacterial cell wall, and the results presented here are promising and warrant further investigation. Therefore, future studies aimed at assessing and producing clinically feasible sources of GSE for *in vivo* studies are necessary to translate these findings into clinical use. Here, the inhibitory effect of GSE increased with increasing concentration. Similarly, (Xu *et al*. 2007) studied the antibacterial effect of GSE against food-borne pathogens, *L. monocytogenes* and *Salmonella* sp. Furthermore, GSE has been examined against food-borne pathogens *B. subtilis*, *C. albicans*, *E. coli* O157:H7, *P. aeruginosa*, *S. enteritidis*, and *S. aureus* (Cvetnić and Vladimir 2004; Song *et al*. 2019). *K. pneumoniae* is notoriously known for its' ability to form biofilms, which in most cases are the prominent causes of healthcare-associated infections. The formation of biofilms dangerously limit therapy options by colonizing tissues and medical devices, such as catheters enabling the rapid dissemination of antibiotic resistance genes (Surgers *et al*. 2019). In our study, biofilm formation was seen in 81% (n = 9) of *K. pneumoniae* isolates. Our study depicts crystalline colonies-turned black on CRA as an indicator of biofilm formation. However, after treatment with 5% (v/v) GSE, biofilm formation is significantly reduced. Comparably, the wells of the microtiter plate are a surface for biofilm-associated cells to attach to, and as seen by the stained crystal violet, the intensity of the color is an indication of strong biofilm formation. The intensity of the crystal violet is diminished after treatment and hence, depicts the reduction of biofilm formation. These results show that a concentration of 5% GSE was able to inhibit and destabilize the biofilm formation of all isolates tested. According to Song *et al*. (2019), *S. aureus* and *E. coli* biofilms were significantly inhibited by GSE at concentrations above 1/4 × MIC (6.25 μg/mL) and 1/8 × MIC (31.25 μg/mL), respectively.

In the present investigation, the cell morphology of the untreated (control) and treated *K. pneumoniae* cells with GSE was observed. This provides concrete evidence that the presence of 10% GSE causes extreme stress for treated bacterial cells, leading to severe intracellular damage, membrane disruption, cell leakage and eventually, death. One potential hypothesis is that GSE interacts with the membrane of *K. pneumoniae*, causing shrinkage which leads to morphological deformation and leakage of cytoplasmic contents. Similarly, electron micrographs displayed membrane disruption of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* by the action of GSE (Heggers *et al*. 2002). Also, in connection with membrane disruption, the results of this study revealed substantial differences in the amount of leaked proteins in untreated and treated cells. Rapid release of intracellular contents and a considerably significant amount of protein leakage was observed in the case of treated cells. As anticipated, the high amount of proteins in the suspension of treated cells indicates severe damage to the bacterial cell membrane caused by GSE.

The correlation between the production of β-lactamases and the spread of resistance among isolates of the Enterobacteriaceae family is very high (Surgers *et al*. 2019), and in particular, ESBLs (extended-spectrum beta-lactamases) are enzymes that deactivate β-lactam antibiotics by hydrolysis and have the ability to transfer bacterial resistance to the penicillins, first-, second- and third-generation cephalosporins (Paterson and Bonomo 2005). In 1983, the first cases of *K. pneumoniae* strains producing β-lactamase enzymes were reported and are notably deemed responsible for the majority of infections caused by multi-drug resistant strains (Sedighi *et al*. 2017). Additionally, most of the genes encoding for these enzymes are located on highly transferable plasmids that further facilitate antibiotic resistance among members of the Enterobacteriaceae family. Among Gram-negative bacteria and MDR *K.*

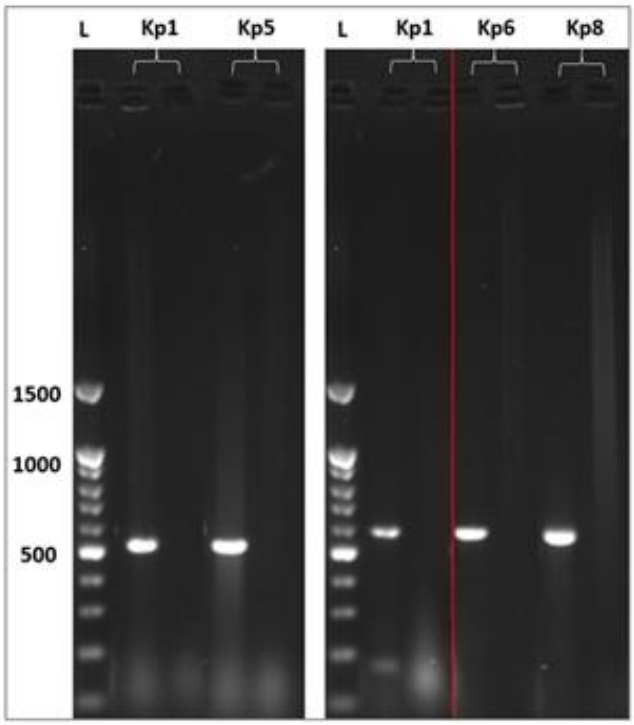


Fig. 3: A representative picture of PCR amplification products using primers for *bla*$_{TEM}$ and *bla*$_{CTX}$ for *K. pneumoniae* extracted DNA. Left gel, isolates Kp1 and Kp5 with *bla*$_{TEM}$ primer (before and after treatment with GSE); whereas, right gel shows isolates Kp1, Kp6, and Kp8 with *bla*$_{CTX}$ primer (before and after treatment with GSE). The results of isolate Kp1 in the right gel was taken from a separate gel and combined in this picture

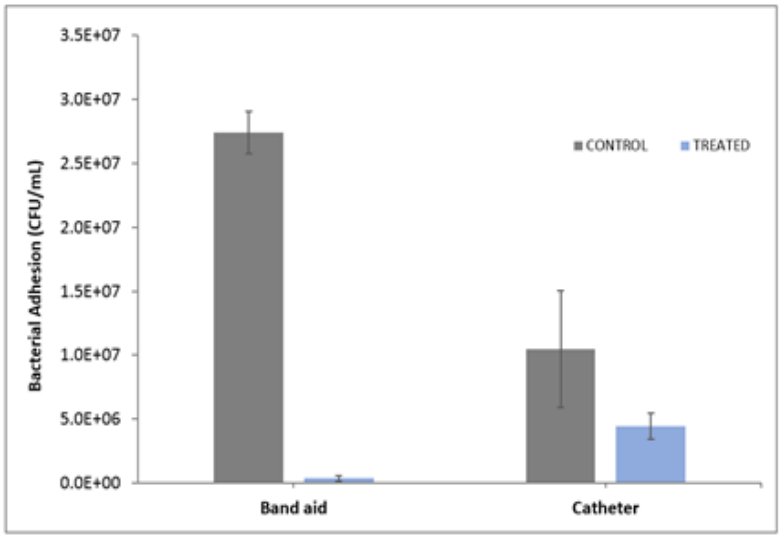


Fig. 4: Anti-adhesion effect of GSE on *K. pneumoniae* isolates on both Band-Aids and catheters. The bars represent a mean of three data points and SD (Mean ± SD)

pneumoniae, three major ESBLs are notably of high importance and concern. These include TEM, SHV and CTX-M types (Bora *et al.* 2014; Memariani *et al.* 2015; Veras *et al.* 2015; Mobasseri *et al.* 2019; Mondal *et al.* 2019). The β-lactamase TEM genes are highly predominant among Gram-negative bacteria, with the first case of *bla*$_{TEM-1}$ reported from an *E. coli* isolate in Athens, Greece, and named according to the patients' name, Temoneira (Paterson and Bonomo 2005). Simultaneously, *bla*$_{CTX}$ genes are named so for their potent ability to hydrolyze

cefotaxime, while bla_{TEM} are highly effective against ampicillin and extended-spectrum cephalosporins containing oxyimino-*β*-lactams side groups such as ceftazidime, cefotaxime, and aztreonam (Paterson and Bonomo 2005; Castanheira *et al*. 2008; Ramirez *et al*. 2019). In this context, this is the first study investigating the mode of action of GSE against plasmids harboring two predominant genes in 11 *K. pneumoniae* clinical isolates, bla_{CTX-M}, and bla_{TEM-1}, encoding the *β*-lactamase enzymes in the Kurdistan region of Iraq. The most notable finding of this study highly supports our hypothesis and demonstrates GSE's ability in efficiently degrading plasmids harboring these antibiotic-resistant genes. Simultaneously, after treatment with 10% of GSE, DNA and RNA of *K. pneumoniae* showed significant reduction (data not shown), while plasmids were completely sheared and degraded. In accordance to our results, other studies also demonstrate bla_{CTX-M} as being the dominant ESBL gene type (Castanheira *et al*. 2008; Bora *et al*. 2014; Lin *et al*. 2016; Ramirez *et al*. 2019). Worldwide dissemination of the bla_{CTX-M} gene can be seen across continents such as in Asia (Lee *et al*. 2011; Zhang *et al*. 2016; Runcharoen *et al*. 2017; Kim and Ko 2019; Xu *et al*. 2019), Africa (Storberg 2014; Agyekum *et al*. 2016), and the Americas (Ramirez *et al*. 2019; Rocha *et al*. 2019). Additionally, PCR showed no amplification of the resistant genes in the case of the treated cells with 10% of GSE. This proves that the mechanism of action of only 10% of GSE not only reduces DNA and RNA significantly, but completely degrades plasmids harboring these resistant genes.

For further applications, Band-Aids and catheters were incorporated with GSE to investigate GSE's ability in the reduction of bacterial adhesion. The ability of bacterial pathogens to adhere to host tissue is usually one of the first steps in the formation of biofilms and overall contributes to the pathogenicity of the microorganism (Krachler and Orth 2013). Preventing the initial bacterial attachment to host surfaces is an effective strategy of preventing biofilm formation, and hence, treating bacterial infections (Song *et al*. 2019). In our study, GSE was investigated for its' anti-adhesion activity on the surfaces of Band-Aids and catheters. Additionally, GSE efficiently reduced bacterial adhesion on the surface of Band-Aids; however, no noteworthy results were seen in the case of catheters.

Conclusion

Our data show that GSE exerts strong antimicrobial activity, prevents bacterial growth, inhibits biofilm, causes intracellular damage and cell lysis, and most significantly, effectively destroys virulence plasmids harboring antibiotic-resistant genes, such as those encoding ESBL proteins. This is the first study investigating GSE mode of action against resistance plasmids in *K. pneumoniae*. Our data and results call attention to the importance of investigating these resistance genes that are responsible for the spread of critical nosocomial and drug-resistant infections, where treatment options are exceedingly difficult and limited. Targeting these plasmids that harbor resistance genes could be an effective remedy in the control and surveillance of antibiotic resistance. Further research is strongly recommended to investigate the applications of GSE in treating drug-resistant bacterial infections and becoming an alternative source of antibiotics.

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Author Contributions

This work is a part of an undergraduate project and has been presented at the 2nd World Congress on Undergraduate Research on 23–25 May 2019 at the University of Oldenburg, Germany. Soma Barawi, Rawezh Hamasalih, Aram Mohammed, Barham Abdalrahman, and Salar Abdalaziz were senior students in the Biology Department at the University of Sulaimani at the time of presentation. They have carried out the experiments. Haider Hamzah is their mentor. Haider Hamzah has designed all the experiments and drafted the paper. All authors read and approved the final manuscript.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflict of Interest

The authors declare that they have no competing interests.

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Ethics Approval

Not applicable to this paper

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